GPHE 119304—11/6/2005—SRIVIDYAR—154165

Pharmacy Education, 2005; 00(0): 1–12

Cytochrome P450 1A2 phenotyping for student laboratories

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Abstract

The purpose of this research is to impart basic knowledge about the cytochrome P450 metabolism of xenobiotics and its meaning for pharmaceutical and medical problems by means of experiment-led learning. The teaching approach includes an experimental part and bibliographic investigations as well as a discussion of the obtained results. The experimental work describes two simple methods for phenotyping cytochrome P450 1A2 in humans by HPLC–UV measurement of caffeine and its main metabolite paraxanthine in urine and saliva. These simple and inexpensive methods are applicable to demonstrate the importance of cytochrome P450 enzymes concerning drug inefficacy, drug–drug interactions and adverse drug reactions.

Two simple and fast HPLC–UV methods for phenotyping cytochrome P450 1A2 in humans are presented. Using these methods, probands can be classified into poor and fast metabolisers by the determination of the paraxanthine/caffeine ratio in saliva and urine. Since our HPLC methods are inexpensive and simple to carry out, the experiments are particularly suitable for pharmaceutical and medical student laboratories.

Keywords: *Clinical Pharmacy, CYP1A2, cytochrome P450, HPLC, phenotyping, laboratory teaching*

Introduction

Context and background

Cytochrome P450 (CYP) enzymes represent a "super family" of hemoproteins catalysing the metabolism of a large number of endogenous and exogenous compounds. The isoenzymes are located in various tissues, such as liver, gut, lung, kidneys and intestine. About 90–95% of these enzymes occur in the smooth endoplasmatic reticulum of liver cells (Schrenk Brockmeier, Mörike, Bock and Eichelbaum 1998). Only four to five specific subfamilies seem to be responsible for about 90% of the metabolism of commonly used drugs (Stockley 2001) and CYP3A4 is the predominant isoenzyme involved in the metabolism of xenobiotics followed by CYP2D6, CYP2C19, CYP2C9, and CYP1A2. Table I shows examples of therapeutically used drugs and the corresponding CYP isoenzymes responsible for their metabolism.

Population differences exist due to inter-individual variability of the isoenzymes (Kalow and Tang 1993). Concerning the isoenzyme CYP1A2 these inter-individual differences can be caused by exogenous factors like cigarette smoke, intake of charcoal broiled food or cruciferous vegetables, and drug consumption. In addition, due to genetic polymorphisms the activities of certain isoenzymes (e.g. CYP2D6) are highly variable (Brüggmann 2003). Endogenous factors such as genetic inheritance, body weight or liver disorders also influence the activities of CYP enzymes (Koch, ten Tusscher, Koppe and Guchelaar 1999). For example, certain drugs induce the activity of CYP1A2 by means of an increasing expression of hepatic CYP1A2 (Table I). Consequently, the plasma concentrations of concomitantly administered CYP1A2 substrates may decrease, resulting in an insufficient therapeutic effect. On the other hand, the inhibition of CYP1A2 causes a rise of the drug plasma concentration, resulting in adverse drug reactions or side effects (Table I). Thus, toxic drug interactions are likely to occur, especially if CYP1A2

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ISSN 1560-2214 print/ISSN 1477-2701 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/15602210500193219

Table I. Cytochrome P450 families 1–3 and metabolised drugs.

Family	Subfamily	Gene	Drugs	Inducers	Inhibitors
1	A	2	caffeine amitryptiline theophylline	broccoli, charcoal- grilled meat tobacco smoke	furafylline
2	A	6	nicotine coumarin	tranylcypromine	–
	B	6	cyclophosphamide buproprione	phenobarbital phenytoin	–
	C	8	paclitaxel trimethoprim	–	quercetin
	C	9	diclofenac phenytoin tolbutamide	rifampin	sulfaphenazole
	C	19	omeprazole diazepam warfarine	rifampin	mephenytoin
	D	6	debrisoquine sparteine dextromethorphan	–	quinidine
	E	1	acetaminophen ethanol chloroxazone	ethanol isoniazid	disulfiram
3	A	4	cyclosporine midazolam terfenadine	rifampin phenytoin carbamazepine	ketoconazole

References for cytochrome P450 Drug Interaction Table/Part 1 of 7: 1A2 Isoform (03/15/2004): http://medicine.iupui.edu/flockhart/p450ref6.html#3A457sub
The Cytochrome P-450 Enzyme System (21/12/2003): http://www.edhayes.com/startp450.html

substrates with a narrow therapeutic window, such as theophylline, are involved. The knowledge of the actual CYP1A2 activity of a patient can help to avoid such unwanted effects.

In principle, determination of the activity of CYP enzymes can be performed by two different methods that enable the classification of a population into poor and extensive metabolisers: Genotyping and phenotyping. Genotyping describes the determination of the individual genotype—which is required usually only once in a lifetime—by the identification of specific alleles. In most instances, genetic variations are point mutations known as single nucleotide polymorphisms (SNPs) (Brüggmann 2003, Licino and Wong 2002).

For phenotyping, a marker substance specifically metabolised by the studied CYP enzyme is applied as a single dose. The extent of metabolism of the marker substance is characterised by the metabolic ratio (MR) which is defined by the relation of the unchanged marker concentration to the specific metabolite(s) in serum, plasma, urine or saliva (Brüggmann 2003). The classification into poor and extensive metabolisers was carried out by comparing the evaluated MRs to standard values reported in the literature (Kaddlubar et al. 1990, Butler et al. 1992).

The purpose of this paper is to describe two simple, fast, and efficient methods for the phenotyping of CYP1A2 that catalyses the N-demethylation of caffeine to paraxanthine (see Figure 1). These non-invasive phenotyping methods are based on the HPLC determination of the paraxanthine/caffeine ratio in saliva and urine after oral ingestion of a cup of coffee containing approximately 200 mg caffeine. The major pathway of the biotransformation of caffeine is the metabolism into paraxanthine by CYP1A2 (73–80%), theobromine (10%) and theophylline (10%) (Holland, Godfredsen, Page and Connor 1998). As described in the literature, caffeine is a suitable marker substance for phenotyping CYP1A2 (Bendriss, Markoglou and Wainer 2000, Carillo et al. 2000, Holland, Godfredsen, Page and Connor 1998, Koch et al. 1999, Nyéki, Biollaz, Kesselring and Décosterd 2001). The substance is well-tolerated and shows a fast and complete gastrointestinal absorption, as well as a short half-life time. The dosage of 100–200 mg caffeine has practically no side effects in comparison to other substrates of CYP1A2. This dosage is also suitable for subjects with induced CYP1A2 activity, and leads to measurable urinary paraxanthine and caffeine concentrations three to six hours after caffeine ingestion. The urinary paraxanthine/caffeine ratios correlated well with plasma values at four hours post-dose (Carillo et al. 2000, Crews, Olivier and Wilson 2001, Fuhr and Rost 1994, Fuhr et al. 1996).

Salivary paraxanthine and caffeine concentrations are measurable 2–15 h after administering a dose of

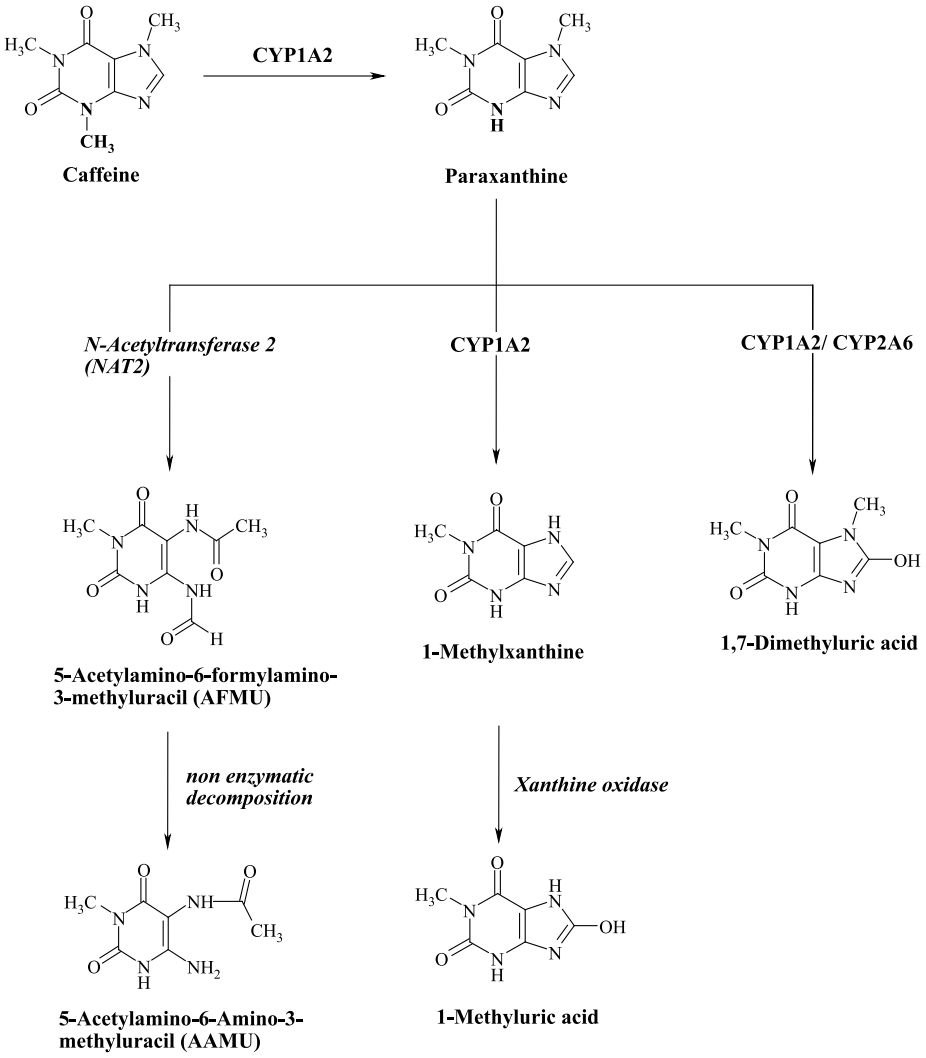

Figure 1. Main pathways of caffeine metabolism in humans adapted by references [Nyéki et al. (2001) and Carillo and Benitez (1994)].

100–200 mg caffeine. Best correlation of salivary paraxanthine/caffeine ratios to plasma values in non-smokers was found 6–10 h post-dose (Spigset, Hägg, Söderström and Dahlquist 1999, Tantcheva-Póor, Zaigler, Rietbrock and Fuhr 1999). Paraxanthine is both a product of caffeine metabolism and a substrate of CYP1A2, therefore small alterations in the sampling time after caffeine ingestion appear to affect the comparability of the paraxanthine/caffeine ratios (Fuhr and Rost 1994, Fuhr et al. 1996, Spigset et al. 1999). Hence the point in time for sample collection was four hours for urine and six hours for saliva after the ingestion of a cup of coffee containing approximately 200 mg caffeine (Tantcheva-Póor et al. 1999).

The HPLC–UV methods were validated in terms of specificity, linearity, accuracy, precision and stability. Therefore, our phenotyping methods are suitable for the assessment of urinary and salivary paraxanthine/caffeine ratios, and for the classification of volunteers into poor and extensive metabolisers regarding CYP1A2 activity.

The teaching approach

The task for the students consists of three parts:

(1) Questionnaire to volunteer characteristics (see Appendix A) and sample collection;
(2) Experimental determination of paraxanthine/caffeine ratios in urine and saliva;
(3) Discussion of the results in comparison to data found in the literature. Furthermore, the results have to be discussed with regard to lifestyle (smoking), medication, diseases, age and gender.

The findings are presented and discussed within the group in a teaching lesson. Thus, basic knowledge of the cytochrome P450 metabolism and its relevance

for drug therapy is imparted by experiment-led learning.

Materials and methods

Chemical reagents:

- methanol
- caffeine, paraxanthine and etofylline
- acetic acid
- ethylacetate
- ultrapure water

Equipment:

- containers for urine collection
- 10 ml glass tubes for saliva collection and sample pretreatment
- pipettes
- disposable pipettes
- disposable gloves
- volumetric flasks
- ultrasonic bath
- centrifuge
- vortex mixer
- HPLC instrument
- SPE Vacuum Manifold
- Strata-X® (60 mg/3 ml) SPE cartridges

Instrumentation and conditions

Analysis of urine samples

Instrumentation. Urine samples were analysed on an Agilent 1100 Series HPLC instrument (Agilent Technologies, Waldbronn, Germany) consisting of a vacuum degasser, a binary pump forming a high pressure gradient by a static mixer (delay volume of 600–900 µl), an autosampler, a thermostated column compartment, an UV diode array detector, and a LC3D Chemstation HP Kayak XM600 equipped with 3DSoftware® (Version 8.04). Analyses were performed using a LiChrospher® 100 RP18e 5 µm LiChrocart® (125 × 4 mm I.D.) HPLC column and a LiChrospher® RP18e 5 µm LiChrocart® (4 × 4 mm I.D.) precolumn (both Merck Eurolab, Darmstadt, Germany). UV detection was performed at 271 nm and the column temperature was set to 20°C. The mobile phase consisted of water (A) and methanol (B). The following gradient was run: Time 0–10 min 10% (B)/ 90% (A), 10–14 min 10 to 30% (B)/ 70% to 30% (A), 14 to 16 min 30% (B)/ 70% (A), 16 to 17 min 30 to 100% (B)/ 70 to 30% (A), 17 to 20 min 100% (B)/ 0% (A), 20 to 21 min 100 to 30% (B)/ 0 to 70% (A) and 21 to 24 min 30% (B)/ 70% (A). The flow-rate was set to 1.5 ml/min and the injection volume was 25.0 µl.

Sample preparation. Two, four and six hours after ingestion of a cup of coffee containing approximately 200 mg caffeine, spot urine samples were collected and centrifuged at 10,000 rpm (Eba 12, Hettich, Kirchlengern, Germany) for 5 min at ambient temperature; 25.0 µl of the supernatant were injected onto the HPLC column without further sample preparation.

Analysis of saliva samples

Instrumentation. Solid phase extraction (SPE) was performed on a Vacuum manifold (Visiprep™, Supelco (Taufkirchen, Germany). Strata-X® SPE cartridges (60 mg/3 ml) were supplied from Phenomenex (Aschaffenburg, Germany).

Saliva samples were analysed on a Varian HPLC system (Varian GmbH Deutschland, Darmstadt, Germany) equipped with two isocratic pumps and an external gradient mixer, a Prostar 320 UV-Vis detector and a Prostar 410 autosampler. The separation was performed using the aforementioned HPLC column. UV detection was performed at 271 nm and the temperature of the column oven was set to 25°C. Isocratic elution was achieved with methanol-(1% acetic acid in water) 12:88 (v/v). The flow-rate was set to 2.0 ml and the injection volume was 25 µl. Data collection and integration was accomplished using Varian Star® workstation Version 6.0.

Sample preparation. Spot saliva samples were collected two, four, six and eight hours after ingestion of a cup of coffee (Tantcheva-Póor et al. 1999). Before analysis, the saliva samples were centrifuged at 12,000 rpm (Eba 12, Hettich, Kirchlengern, Germany) for 10 min at ambient temperature; 0.9 ml of the supernatant was transferred to a volumetric flask. After addition of 0.1 ml of the internal standard (10.0 µg/ml etofylline in water) the sample was diluted with water to 5.0 ml, vortexed for 30 s and an aliquot of 4 ml was applied to the SPE cartridge, preconditioned with 2 ml methanol and 2 ml water. The cartridge was washed with 2 ml water and dried by sucking air through the cartridge for 1 min. Subsequently, caffeine and paraxanthine were eluted using 2 ml methanol followed by 1 ml methanol-ethylacetate 70:30 (v/v). After evaporation of the eluate to dryness under a gentle stream of nitrogen at 25°C, the residue was reconstituted in 100 µl methanol -(1% acetic acid in water) 12:88 (v/v), transferred into HPLC screw cap vials and analysed by HPLC.

Stock solutions

Stock solutions containing 1 mg/ml caffeine and 1 mg/ml paraxanthine were prepared in methanol/ water 30:70 (v/v) and diluted with water to obtain standard solutions at concentrations of 250.0/150.0/ 100.0/50.0/25.0/15.0/5.0/3.0/1.0 µg/ml of each

substance. The internal standard etofylline was prepared by the dilution of the etofylline stock solution (1 mg/ml in methanol/water 30:70 (v/v)) with water to obtain a concentration of 10.0 μg/ml. The salivary calibration standards were prepared by transferring 1.0 ml of caffeine and 1.0 ml paraxanthine standard solution, respectively, into a volumetric flask and adding water to 10.0 ml to obtain concentrations of 0.1/0.5/2.5/5.0/10.0/25.0 μg/ml of each. Quality control (QC) samples were prepared by spiking blank saliva with standard solutions of caffeine and paraxanthine to obtain 0.3/ 1.5/15.0 μg/ml of each.

For urinary CYP1A2 phenotyping, calibration standards were prepared by transferring 1.0 ml of a caffeine and paraxanthine stock solution into a volumetric flask and adding water to 10.0 ml to obtain concentrations of 0.25/0.5/5.0/10.0/12.5/ 25.0 μg/ml. QC samples were prepared by spiking blank urine with three concentrations of standard solutions, containing both caffeine and paraxanthine, to obtain 1.0/7.5/15.0 μg/ml of each.

Stability of samples and solutions

Calibration standards and urine samples are stable at room temperature for two days and should be stored at 2–8°C until use. Stock solutions and saliva samples should be kept at −20°C.

Method validation for CYP1A2 phenotyping using saliva

Specificity and selectivity were investigated by analysis of pooled blank saliva of four healthy volunteers being on a caffeine-free diet for at least 72 h. Blank saliva used for validation was checked for the absence of caffeine and paraxanthine and for possible interferences from endogenous compounds. Saliva was spiked with different standard solutions of caffeine and paraxanthine to give calibration standards ranging from 0.1–25.0 μg/ml, which were analysed by SPE-HPLC ($n = 6$). Correlation coefficients and calibration equations were assessed by linear regression analysis. Calibration curves were generated by plotting average peak area ratios of analyte/I.S. ($n = 6$), vs. respective nominal concentrations of each analyte. Intraday precision and accuracy were evaluated from the results of the QC samples processed at the same day (0.3/ 1.5/ 15.0 μg/ml of caffeine and paraxanthine in saliva; $n = 6$). Inter-day variability was assessed by analysis of the QC samples ($n = 6$) at different days. According to the Guidance for Industry-Bioanalytical Method validation (2001), recoveries were calculated from average peak area ratios ($n = 6$) obtained after analyses of three quality control samples of caffeine and paraxanthine (0.3/ 1.5/ 15.0 μg/ml) in saliva and corresponding standard solutions (3.0/15.0/150 μg/ml caffeine and paraxanthine in water).

Method validation for CYP1A2 phenotyping using urine

In order to prove specificity and selectivity of the method, pooled blank urine of four healthy volunteers was spiked with paraxanthine and caffeine and analysed by HPLC. Linearity was assessed by spiking blank urine with caffeine and paraxanthine standard solutions to give calibration standards at different concentrations ranging from 0.25–25.0 μg/ml of each substance by means of HPLC analysis ($n = 6$). Calibration curves were constructed by plotting average peak areas of the analyte against nominal concentrations. QC samples at three concentrations (1.0/ 7.5/ 15.0 μg/ml of caffeine and paraxanthine in urine) were analysed with HPLC ($n = 6$) for the determination of intra- and inter-day precision and accuracy. Stability of caffeine and paraxanthine in blank urine was assessed by storing the quality control samples at various conditions (light exposure, room temperature, 2–8 °C, −20°C) for 1, 3 and 7 days, as well as for 4 and 12 weeks.

Determination of paraxanthine/caffeine ratios in urine and saliva

Preparation of calibration curves. In urine, the calibration standards containing 0.25/ 0.5/ 5.0/ 10.0/ 12.5/ 25.0 μg/ml of caffeine and paraxanthine were analysed by HPLC ($n = 6$) and calibration curves were assessed by plotting average peak area ratios of the analyte ($n = 6$) vs. the corresponding nominal concentration. The salivary calibration standards (0.1/ 0.5/ 2.5/ 5.0/ 10.0/ 25.0 μg/ml of caffeine and paraxanthine in saliva) were analysed by SPE-HPLC ($n = 6$). The calibration curves were assessed by plotting average peak area ratios of analyte/I.S. ($n = 6$) vs. the corresponding nominal concentrations of the respective analyte.

Calculation of caffeine and paraxanthine contents and the paraxanthine/caffeine ratios in urine and saliva. The caffeine and paraxanthine contents in urine and saliva were calculated using the obtained calibration equations and the determined peak areas in urine and the peak area ratios of analyte/I.S. in saliva, respectively (see *Preparation of calibration curves*). Representative chromatograms of blank urine and spot urine samples are shown in Figure 2A and B, HPLC chromatograms of saliva samples collected after caffeine ingestion in Figure 3.

Each paraxanthine/caffeine ratio was calculated using the obtained caffeine and paraxanthine contents of the corresponding urine or saliva samples.

Data analysis

Linear regression, analyses of the calculation of caffeine and paraxanthine contents, and going into

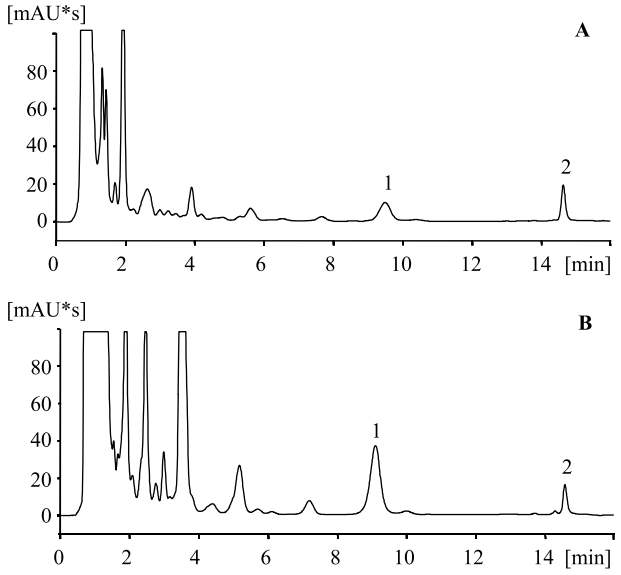

Figure 2. (A) HPLC chromatogram obtained from the spot urine sample of a non-smoker; (1) caffeine and (2) paraxanthine. For experimental details see Materials and methods. (B) HPLC chromatogram of a spot urine sample obtained from a smoker; (1) caffeine and (2) paraxanthine. For experimental details see Materials and methods.

the merits of linearity, precision, accuracy and recovery were performed using Microsoft® Excel 97 version SR-2. The distribution characteristics of the paraxanthine/caffeine ratios were investigated by the Kolmogorov-Smirnov Goodness of Fit Test using GraphPad Prism® version 2, and differences of means were analysed by the Student *t*-test using Microsoft® Excel 97 version SR-2.

Disposal of waste

The waste mainly consists of water and methanol. Therefore, it can be disposed into the organic halogen-free waste. Disposable gloves and pipettes can be kept in plastic bags and taken to the litter.

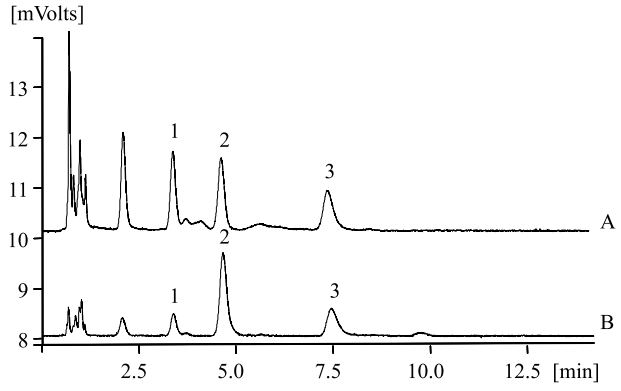

Figure 3. (A) HPLC chromatogram of a saliva sample obtained from a smoker and (B) from a non-smoker 6 h after caffeine ingestion; (1) paraxanthine, (2) internal standard and (3) caffeine. For experimental details see Materials and methods.

Results

Method development and validation of the HPLC–UV determination of paraxanthine and caffeine in saliva

Due to the high viscosity of saliva, the samples had to be diluted with water, and the internal standard etofylline was added prior to solid phase extraction. With respect to caffeine, paraxanthine and etofylline recovery, the elution was carried out with methanol/ethylacetate in order to obtain good recoveries for all substances. By evaporation and reconstitution in the mobile phase, the saliva samples could be tenfold concentrated. Since the purified saliva extracts contained only a few interfering substances, a baseline separation of the analyte peaks within 8 min was obtained using isocratic elution with methanol-1.0% acetic acid 12:88 (v/v) (Figure 2).

In addition, a good peak shape, an appropriate analysis time and a good selectivity and resolution were achieved. Considering the maxima of absorbance of the methylxanthine compounds in the mobile phases, the detection wavelength was set to 271 nm.

Method validation was carried out using pooled blank saliva of four volunteers spiked with caffeine and paraxanthine. The corresponding HPLC analyses displayed in Figure 3 did not show any interference. Validation data are summarized in Table II. Linearity was obtained in the range of 0.1 to 25.0 μg/ml for paraxanthine ($y = 0.631x + 0.0438$; $r = 0.999$) and caffeine ($y = 0.6097x + 0.0179$; $r = 0.999$). Accuracy was between −1.6% and −14.9% for caffeine and −3.5% and −4.7% for paraxanthine. Precision was between 3.0% and 14.6% for caffeine, and between 2.5% and 14.5% for paraxanthine. Since the intra- and inter-day precision and accuracy are less than ±15.0%, the method can be considered to be acceptable. The limit of detection based on a signal-to-noise ratio (S/N) of 3 was 0.06 μg/ml for caffeine and 0.05 μg/ml for paraxanthine. The limit of quantification (S/N = 5) (Guidance for Industry-Bioanalytical Method validation 2001) for caffeine was 0.09 μg/ml and 0.10 μg/ml for paraxanthine. Recoveries were calculated from average peak area ratios ($n = 6$) obtained from analyses of the three quality control samples and the corresponding caffeine and paraxanthine standard solutions in water. Recoveries were between 91.1% and 96.0% for caffeine and between 96.7% and 101.4% for paraxanthine (Table II). Recovery of the I.S. etofylline was 96.3% ($n = 6$).

Due to reproducible results of the validation, the SPE-HPLC method is suitable to determine caffeine and paraxanthine in saliva.

Table II. Validation results of the HPLC determination of paraxanthine and caffeine in saliva.

	Caffeine		Paraxanthine	
	QC	C.V.	QC	C.V.
Intraday precision ($n = 6$)	0.3 μg/ml	14.34%	0.3 μg/ml	14.48%
	1.5 μg/ml	3.99%	1.5 μg/ml	5.48%
	15.0 μg/ml	2.98%	15.0 μg/ml	3.47%
Calibration regression	$y = 0.6323x + 0.0293$		$y = 0.6566x + 0.0163$	
Correlation coefficient	$r = 0.9997$		$r = 0.9998$	
Interday precision ($n = 6$)	0.3 μg/ml	14.63%	0.3 μg/ml	14.34%
	1.5 μg/ml	5.27%	1.5 μg/ml	5.33%
	15.0 μg/ml	3.34%	15.0 μg/ml	3.93%
Calibration regression	$y = 0.6304x + 0.0284$		$y = 0.6084x + 0.0215$	
Correlation coefficient	$r = 0.9995$		$r = 0.9995$	
Accuracy ($n = 6$)	0.3 μg/ml	−14.91%	0.3 μg/ml	−14.97%
	1.5 μg/ml	−9.86%	1.5 μg/ml	−9.66%
	15.0 μg/ml	−1.64%	15.0 μg/ml	−4.73%
Recovery ($n = 6$)	0.3 μg/ml	91.13%	0.3 μg/ml	96.66%
	1.5 μg/ml	94.44%	1.5 μg/ml	98.46%
	15.0 μg/ml	96.02%	15.0 μg/ml	101.39%

C.V. = coefficient of variation, QC = Quality Control Sample

Method development and validation of the HPLC–UV determination of caffeine and paraxanthine in urine

For this application, the urine was directly injected onto the column after centrifugation. A baseline separation of paraxanthine and caffeine in urine was obtained within 15 min (Figure 4). The injection volume was 25.0 μl and the detection wavelength was 271 nm.

No interferences with endogenous compounds were detected in pooled blank urine samples of four individuals (Figure 5A). For intra- and inter-day precision and accuracy, the above-mentioned criteria were applied. Validation data are shown in Table III. The limit of detection (S/N = 3) was 0.11 μg/ml for caffeine and 0.15 μg/ml for paraxanthine, the limit of quantification (S/N = 5) for caffeine 0.20 and 0.25 μg/ml for paraxanthine. Samples stored at daylight and in the dark were stable at +20°C for at least one week. At +8°C samples showed good stability for at least four weeks. Stability for at least 12 weeks was obtained for caffeine and paraxanthine in urine samples stored at −20°C. Thus, the described HPLC method proved to be effective for the

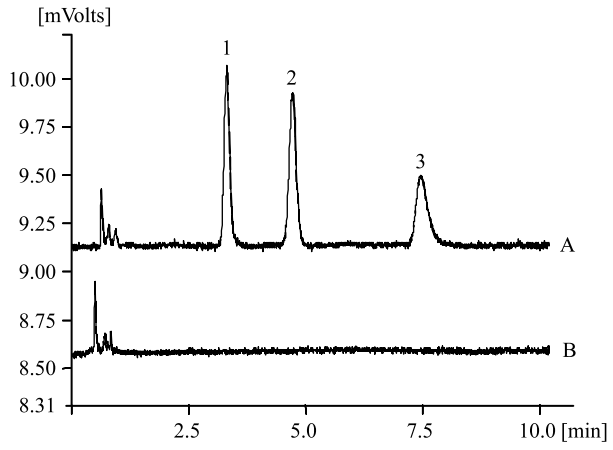

Figure 4. (A) HPLC chromatogram obtained from blank urine after HPLC analysis. For experimental details see Materials and methods. (B) HPLC chromatogram of a blank urine spiked with (1) caffeine and (2) paraxanthine (5.0 μg/ml of each) after HPLC analysis. For experimental details see Materials and methods.

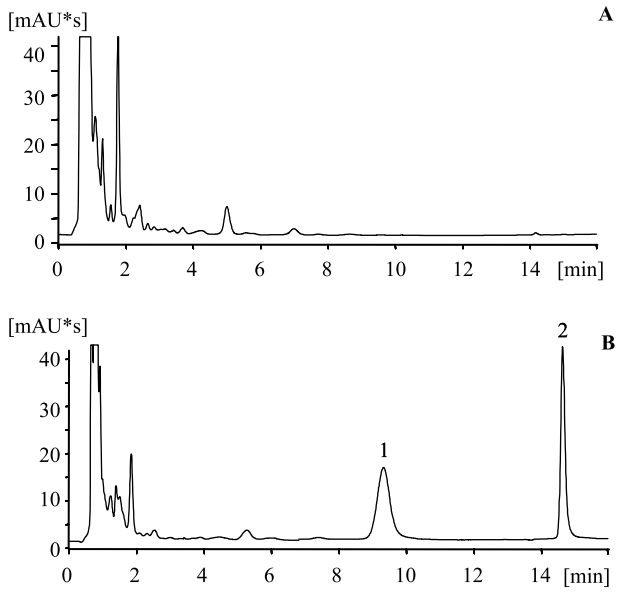

Figure 5. (A) HPLC chromatogram obtained from a blank saliva spiked with (1) paraxanthine (1.5 μg/ml), (2) etofylline (1.5 μg/ml) and (3) caffeine (1.5 μg/ml) and (B) HPLC chromatogram obtained from a blank saliva after SPE-HPLC analysis. For experimental details see Materials and methods.

Table III. Validation results of the HPLC determination of paraxanthine and caffeine in urine.

	Caffeine		Paraxanthine	
	QC	C.V.	QC	C.V.
Intraday precision ($n = 6$)	1.0 μg/ml	1.52%	1.0 μg/ml	0.67%
	7.5 μg/ml	0.34%	7.5 μg/ml	0.90%
	15.0 μg/ml	0.48%	15.0 μg/ml	1.05%
Calibration regression	$y = 48.043x - 0.3661$		$y = 48.841x + 1.1346$	
Correlation coefficient	$R = 1.0000$		$r = 1.0000$	
Interday precision ($n = 6$)	1.0 μg/ml	1.55%	1.0 μg/ml	1.03%
	7.5 μg/ml	1.36%	7.5 μg/ml	2.06%
	15.0 μg/ml	1.13%	15.0 μg/ml	2.08%
Calibration regression	$y = 47.705x - 0.9618$		$y = 49.283x + 1.7914$	
Correlation coefficient	$r = 0.9999$		$r = 1.0000$	
Accuracy ($n = 6$)	1.0 μg/ml	+0.73%	1.0 μg/ml	+1.21%
	7.5 μg/ml	−0.16%	7.5 μg/ml	+0.28%
	15.0 μg/ml	+0.23%	15.0 μg/ml	+1.09%

C.V. = coefficient of variation, QC = Quality Control Sample.

determination of the metabolic paraxanthine/caffeine ratio in urine.

CYP1A2 phenotyping in saliva and urine

The paraxanthine/caffeine ratios of 12 healthy volunteers were determined in saliva. Table IV summarizes the volunteers' paraxanthine/caffeine ratios six hours after caffeine ingestion in addition to medication, smoking habits, sex, age and body-mass-index (BMI). The collective of volunteers consisted of three male smokers and four male non-smokers, as well as five female non-smokers aged 23–30 years. The BMIs ranged from 19–25. For all saliva samples collected six hours after caffeine intake the obtained ratios ranged between 0.01 and 0.91 (mean: 0.20 ± 0.24). Our examination showed higher paraxanthine/caffeine ratios in male probands (mean = 0.30 ± 0.28, range 0.12–0.91, $n = 7$), male non-smokers (mean = 0.23 ± 0.10, range 0.14–0.37, $n = 4$) and in a heavy smoker (paraxanthine/caffeine ratio = 0.91), in comparison to female non-smokers (mean = 0.07 ± 0.06, range 0.01–0.15, $n = 5$), weak male smokers (mean = 0.13 ± 0.06, range 0.12–0.13, n = 2), and male non-smokers (mean = 0.23 ± 0.10, range 0.14–0.37, $n = 4$) (Figure 6).

The Student *t*-test showed no significance in the differences of mean values: Smokers versus non-smokers ($P < 0.001 = 4.587$; *P*-value: 0.3522), degree of freedom (df = 10), males versus females ($P < 0.001 = 4.587$; *P*-value: 0.0179; df = 10), and male non-smokers versus female non-smokers ($P < 0.001 = 5.408$; *P*-value: 0.1101; df = 7). The metabolisers were classified using the borderline mean paraxanthine/caffeine ratio of 0.30, which was introduced by Tantcheva-Póor et a1. (1999), into ten poor metabolisers (paraxanthine/caffeine ratio ≤0.30) and two extensive metabolisers (paraxanthine/caffeine ratio ≥0.30).

Fifty-two healthy volunteers were examined by the urinary CYP1A2 phenotyping. The group of volunteers consisted of 21 women and 31 men, including

Table IV. Results of the salivary determination of paraxanthine/caffeine ratios in volunteers.

Volunteer	Paraxanthine/Caffeine Ratio 6 h postdose	Age	Sex	BMI	Smoking habits	Medication
1	0.91	27	m	19	+++	–
2	0.13	25	m	25	+	–
3	0.12	n.r.	m	n.r.	+	–
4	0.37	30	m	21	–	–
5	0.23	n.r.	m	n.r.	–	–
6	0.17	24	m	21	–	–
7	0.14	23	m	21	–	–
8	0.15	n.r.	f	n.r.	–	oral contraceptives
9	0.13	23	F	22	–	oral contraceptives
10	0.05	24	f	22	–	oral contraceptives
11	0.02	23	f	20	–	oral contraceptives
12	0.01	27	f	23	–	oral contraceptives

m = male; f = female; n.r. = not reported.

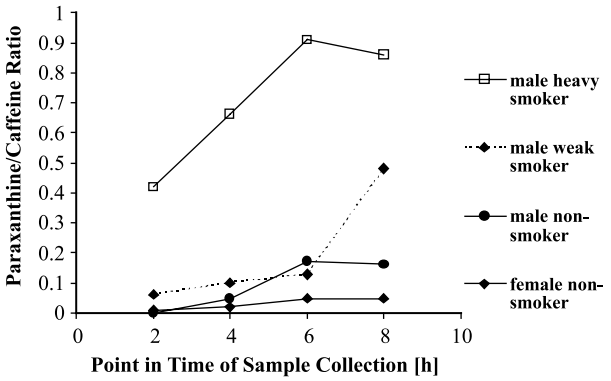

Figure 6. Salivary Paraxanthine/Caffeine Ratios related to the Point in Time of Sample Collection.

8 male smokers and 23 male non-smokers, as well as 6 female smokers and 15 female non-smokers at the age of 21–38 years. The BMIs ranged from 18–29. The mean of the paraxanthine/caffeine ratios was 2.33 ± 2.01. The paraxanthine/caffeine ratios ranged from 0.05–9.01 in women ($n = 21$) and from 0.09–6.40 in men ($n = 31$). The obtained ratios ranged between 0.05 and 9.01 for non-smokers ($n = 38$) and between 0.09 and 6.40 for smokers ($n = 14$). In our examination, males (mean: 2.43 ± 1.68) and smoking volunteers (mean: 2.67 ± 1.93) showed higher mean paraxanthine/caffeine ratios than females (mean: 2.19 ± 2.46) and non-smokers (mean: 2.21 ± 2.05). Differences of mean values were analysed by the Student *t*-test and showed no significant differences: Smokers versus non-smokers (*P*-value: 0.4569; df = 50), males vs. females (*P*-value: 0.6920; df = 50), male non-smokers versus female non-smokers (*P*-value: 0.7504; df = 36) and male smokers versus female smokers (*P*-value: 0.7905; df = 12). Referring to Kaddlubar et al. (1990), Lampe et al. (2000), urinary paraxanthine/caffeine ratios <2.5 characterise poor metabolisers whereas ratios >2.5 characterise extensive metabolisers. According to this classification, 33 healthy volunteers were poor metabolisers and 18 volunteers were extensive metabolisers. One individual showed a ratio of 2.47.

Discussion

The experimental determination of the paraxanthine/caffeine ratios included the treatment of saliva or urine samples, the analysis of the samples with HPLC and finally calculation of the paraxanthine/caffeine ratios using the peak area ratios of the internal standard and the analytes. This experiment-led learning imparted knowledge about the handling of potentially infectious samples, the preparation of standards and calibration samples by handling exact volumes of stock solutions, the application of modern computer hardware and software for the running of HPLC analysis and basic mathematical calculations (e.g. linear regression analysis) for the determination of the paraxanthine/caffeine ratios. These skills are becoming increasingly important for students and pharmacists working in the pharmaceutical industry, and hospitals in particular. *In vivo* phenotyping studies are an integral part of clinical pharmacy and an important aspect of the pharmaceutical education. Since the impact of clinical pharmacy for the pharmaceutical attendance is substantial, the above-mentioned skills strongly contribute to the education of pharmacy students in the field of clinical pharmacy.

The results of our phenotyping studies are discussed in detail in the following paragraphs. Previous studies of Tantcheva-Póor et a1. (1999), Spigset et al. (1999) revealed mean paraxanthine/caffeine ratios of 0.30 ± 0.31 ($n = 786$ healthy (non)-smokers ≥20 years) and 0.70 ± 0.18 (range 0.45 to 1.01, $n = 12$ healthy male non-smokers, 23.6 ± 2.3 years). Male individuals showed higher ratios than females (0.41 ± 0.47 ($n = 371$) vs. 0.07 ± 0.56 ($n = 94$)). In addition, non-smokers (mean = 0.15 ± 0.45, $n = 401$) had lower mean values than heavy smokers (mean = 0.63 ± 0.48, $n = 70$), but did not show significantly different paraxanthine/caffeine ratios in comparison to weak smokers (mean = 0.15 ± 0.51, $n = 85$).

However, the influence of smoking appears to depend on the extent of tobacco consumption (Fuhr and Rost 1994, Fuhr et al. 1996, Carillo and Benitez 1996, Forsyth et al. 2000, Newton et al. 1981, Schrenk et al. 1998, Spigset et al. 1999, Tantcheva-Póor et a1. 1999). Due to a higher activity of CYP1A2 for smokers, the paraxanthine/caffeine ratio is increased in comparison to non-smokers. As can be seen in Figure 6, which summarizes the study on salivary paraxanthine/caffeine ratios, male smokers have significant higher ratios than male non-smokers, and male non-smokers have a slightly higher (not significant) ratio than female non-smokers, which is in agreement with the study of Tantcheva-Póor et a1. (1999). Hence, the CYP1A2 phenotyping in saliva presented here is suitable for the use in student laboratories.

Furthermore, the application of this phenotyping method is an easy and practicable way to demonstrate that tobacco, as a widely used natural stimulant, can lead to an increase in the metabolism of a great variety of synthetic drugs which are metabolised by CYP1A2. Since CYP1A2 is also involved in the oxidative metabolism of freely available Over-The-Counter medicines, such as the analgetic drugs naproxen and acetaminophen (Minors, Coulter, Tukey, Veronese and Birkett 1996, Raucy, Lasker, Lieber and Black 1989); drug toxicity or drug interactions caused by an

inhibition or induction of CYP1A2 are not restricted to drugs only available through prescription for patients with chronic diseases, such as asthma. Acetaminophen, for example, is metabolised via CYP1A2 to N-acetyl-p-benzoquinone imine, an electrophilic and hepatotoxic metabolite (Raucy et al. 1989). This reactive metabolite is also formed via the alcohol-inducible cytochrome P450 enzyme CYP2E1 (Raucy et al. 1989). Since students regularly consume alcohol, acetaminophen and tobacco, they will be highly motivated to understand the coherence between the consumption of lifestyle drugs (e.g. caffeine, tobacco, alcohol), CYP1A2 activity and the potential consequences for health. These important aspects should be discussed in the teaching lessons. Of course, these considerations are also relevant for the advice of patients in pharmacies because their lifestyle can strongly influence the bioavailability of drugs. Thus, pharmacy students should be able to evaluate the factors which can influence the plasma levels of drugs metabolised via CYP1A2 in particular.

Evaluating urine, Kaddlubar et al. (1990) found paraxanthine/caffeine ratios ranging from 0.359–10.621 ($n = 30$, subgroups not specified), and Lampe et al. (2000) a mean ratio of 2.6 ± 0.25 in 17 female and 19 male non-smokers. Sinués et al. (1999) subdivided into non-smokers (0.50–6.05; $n = 95$) and smokers (0.90–21.24; $n = 66$), and McQuilkin, Nirrenberg and Bresnick (1995) divided males (range: 2.70–11.24; $n = 10$) and females (range: 2.19–14.04; $n = 9$). In our investigations, gender has only a small and insignificant effect on the paraxanthine/caffeine ratios, and thus on CYP1A2 activity, because the inter-individual variability was much larger than the sex-related differences. According to Tantcheva-Póor et al. (1999), there is a relevant implication of caffeine intake to enzyme activity due to high daily coffee consumption, whereas methylxanthine-containing food and beverages have less influence on CYP1A2 activity. Our examination showed higher urinary paraxanthine/caffeine ratios in regular heavy coffee drinkers (according to the volunteer's statement of drinking at least 700 ml coffee per day) in comparison to non-coffee drinkers. Due to the homogeneity of the groups and the very small group sizes, no correlation between age or BMI and CYP1A2 activity could be found. A correlation of certain medications, such as oral contraceptives, was not examined systematically in this study because the information about the application of drugs was provided voluntarily and was only used for the discussion of single paraxanthine/caffeine ratios within a teaching lesson.

However, as can be seen in Figure 7, the paraxanthine/caffeine ratio is strongly dependent on the time point of measurement: Sinués et al. (1999) had chosen an interval of 4–5 h for the spot urine sample collection, with the aim to minimize the influence of paraxanthine conversion to secondary metabolites, and found significantly higher mean values for smokers (4.50 ± 3.61; $n = 66$), in comparison to non-smokers (2.57 ± 1.25; $n = 95$). Hence, sample collection of this study was fixed at 4 h post-dose. The resulting mean paraxanthine/caffeine ratios of smokers were higher than in non-smokers, which is in agreement with the results obtained from saliva studies. However, spot urine sample collections 4 h after caffeine intake did not result in reliable classifications of smoking individuals, because the ratios of several smoking individuals were considerably decreased. Moreover, we found one heavy smoker having a urinary paraxanthine/caffeine ratio of 0.09, which normally indicates a poor metaboliser (Carillo and Benitez 1996, Kalow and Tang 1993). This person can be regarded as an outlier.

In principle, high standard deviations were caused by the inter-individual variability, predefined by the genotype, and the intra-individual variability of each volunteer, which was assessed by McQuilkin et al. (1995) to be very different to some extent, particularly caused by nutrition, lifestyle, short-time medication and environmental influences (Lampe et al. 2000). Due to the random choice of volunteers, the determination of CYP1A2 activity just at one day for each volunteer, and unequally distributed subgroups in our study, the standard deviations were higher in comparison to that reported in the literature (Lampe et al. 2000).

We feel that the interindividual variation of results should be extensively discussed in the teaching lessons because pharmacotherapy is often not related to interindividual conditions, which can lead to fatal drug toxicity or drug interactions. In this regard it is important to underline the relevance of genotyping and phenotyping studies in order to evaluate the susceptibility of patients for a specific medication. Since this aspect of pharmacotherapy will be of great

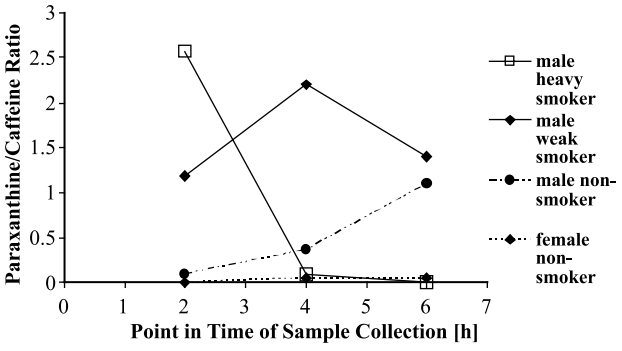

Figure 7. Urinary Paraxanthine/Caffeine Ratios related to the Point in Time of Sample Collection.

interest in the near future, our here presented phenotyping study for the determination of CYP1A2 activity might be a helpful method for the teaching of clinical aspects in the education of pharmacy students.

Taken together, the methods presented here allow the simple and inexpensive determination of caffeine and paraxanthine in saliva and urine. Especially suitable is the CYP1A2 phenotyping in saliva to impart basic knowledge about the cytochrome P450 system, its metabolic pathways, and allows a classification of the volunteers into poor and extensive metabolisers.

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Appendix A: Questionnaire for Volunteers taking part in CYP1A2 Phenotyping

Volunteer No.: Date:

Time point of caffeine ingestion:
Time point of sample collection:

Age:
☐ male ☐ female
Height [m]: Weight [kg]: BMI:

Administered medication(s)?
☐ no ☐ yes

Severe or chronic disease(s)?
☐ no ☐ yes

Smoking Habits?
☐ no ☐ yes

Regular caffeine or methylxanthine intake?
☐ no ☐ yes